*LABELING OF PROTEINS BY ISOTOPIC AMINO ACID DERIVATIVES**

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Previous publications[1-5] from this laboratory have described the catalysis, by intracellular proteolytic enzymes, of transamidation reactions of the type RCO—NH_2 + $NH_2X \rightleftharpoons$ RCO—NHX + NH_3, where the first reactant is a suitable substrate (e.g., glycyl-L-tyrosinamide for cathepsin C) and the second reactant ("replacement agent") is a peptide (e.g., L-leucylglycine) or hydroxylamine. It has been postulated[6] that in such reactions the substrate combines with the enzyme to form an intermediate acyl-enzyme (RCO—E), which then reacts either with water, resulting in hydrolysis, or with NH_2X, resulting in transamidation.[7] The experiments reported in the present communication indicate that insulin and the proteins of rat liver mitochondria can also serve as replacement agents in enzyme-catalyzed transamidation reactions. In these experiments, C^{14}-labeled tyrosine derivatives were employed, and their "incorporation" into the above proteins was measured. Because of the suggestion that aminoacyladenylates are intermediates in the biosynthesis of proteins,[8-11] such C^{14}-labeled compounds also were synthesized, and their "incorporation" into the mitochondrial proteins was examined.

Labeling of Insulin by Isotopic Glycyltyrosinamide.—Glycyl-L-tyrosinamide (GTA) is a synthetic substrate for the intracellular proteinase cathepsin C,[12] which has been partially purified from beef spleen and which specifically catalyzes hydrolytic and replacement reactions involving dipeptide amides or dipeptide esters.[4, 13] C^{14}-labeled GTA acetate was prepared in the manner described previously[4]; uniformly labeled L-tyrosine (18,000 cpm/μmole) was used as starting material. In a typical experiment with insulin as the replacement agent, the incubation mixture (2 ml.) contained: 5 mg. C^{14}-GTA (16.5 μmoles; 297,000 cpm); 60 mg. insulin (Lilly); cathepsin C (0.2 unit; 0.036 mg. protein); cysteine (10 μmoles). The pH was adjusted to 7.0 with NaOH, and the mixture was incubated for 90 minutes at 38° C. During this time, the GTA had been completely deamidated, as shown by parallel measurement of NH_4^+ formation. The incubation mixture was chilled, and 0.66 ml. of 20 per cent trichloroacetic acid was added. The resulting precipitate was centrifuged, washed three times with cold 5 per cent trichloroacetic acid, and dissolved in 2 ml. of cold 0.1 *N* NaOH. Unlabeled glycyl-L-tyrosine (5 mg. in 0.5 ml.) was added, and the dissolved protein was reprecipitated by the addition of 1.8 ml. of 20 per cent trichloroacetic acid, centrifuged, and washed three times with cold 5 per cent trichloroacetic acid. Upon the addition of 2 ml. of ethanol to the centrifuged precipitate, the protein dissolved and was reprecipitated with 4 ml. of ether. The resulting material was washed with ether, dried, and its radioactivity was determined in a gas-flow counter; about 1 mg. was placed on 1-cm.^{2} circles for counting. From the above experiment, 41.6 mg. of protein were obtained; its specific radioactivity was 200 cpm/mg, corrected for self-absorption.

Control experiments in which the enzyme had been omitted or in which GTA had been replaced by labeled glycyl-L-tyrosine (GT) of the same specific radioactivity gave protein samples of negligible C^{14}-content (0 and 5 cpm/mg, respec-

tively). This indicates that the radioactivity "incorporated" into the insulin in the above experiment was not caused by adsorption of unchanged GTA or of GT formed by enzymic hydrolysis. To determine whether the radioactivity was a consequence of the adsorption of the glycyltyrosyl polymer formed by cathepsin C from GTA,[3] control experiments were conducted in which insulin (either untreated or previously incubated with cathepsin C in the absence of GTA) was added to an incubation mixture from which insulin had been omitted at the start. Trichloroacetic acid was added immediately thereafter; the protein sample obtained in the manner described above had a C^{14}-content of 3 cpm/mg. Furthermore, C^{14}-labeled polymer (4,290 cpm/mg) was isolated from an incubation mixture containing isotopic GTA and cathepsin C at pH 7.5 (no insulin present). Upon mixing 1.2 mg. of the labeled polymer with 20 mg. of insulin, followed by the reprecipitation procedure outlined above, a protein sample having 8 cpm/mg was obtained. In addition, mixtures of the polymer and insulin could be separated by paper chromatography, with 2-butanol saturated with 1 per cent acetic acid as the solvent; under the conditions employed in these chromatographic runs, the polymer remains at the origin, and the insulin moves with an R_F of about 0.15. When the labeled insulin was subjected to such chromatographic treatment, all the radioactivity was associated with the insulin spot. It appears unlikely, therefore, that the protein labeled by incubation in the presence of isotopic GTA contained appreciable amounts of adsorbed polymer.

It may be added that, under the conditions employed, the amount of cathepsin C present in the complete incubation mixture was insufficient to cause a detectable hydrolytic cleavage of insulin, as measured by means of a Radiometer autotitrator. Also, separate experiments indicated that the rate of enzymic deamidation of GTA was not appreciably changed by the presence of insulin in the incubation mixture.

To establish whether the glycyltyrosyl residue of GTA had become attached to one of the amino groups of insulin, the labeled protein was subjected to papain digestion[14] for 24 hours, and the incubation mixture was treated with charcoal to adsorb aromatic compounds. Most of the radioactivity could be adsorbed on charcoal, from which it was eluted with ethanol. Paper chromatography of the ethanol extract, with *n*-butanol–acetic acid–water (3:1:1) as the solvent, gave three radioactive ninhydrin-positive components having R_F values of 0.52, 0.70, and 0.86. The component of R_F 0.86 had the greatest C^{14}-content and was investigated further. It migrated as a single spot in several other solvents: in trimethylcarbinol-methyl ethyl ketone-ammonia-water (10:10:3:5) its R_F was 0.43; in 2,6-lutidine-water (65:35) its R_F was 0.13; in pyridine-methanol-water (1:40:10) its R_F was 0.41; and in 2-butanol–formic acid–water (75:15:10) its R_F was 0.18. On acid hydrolysis (6 *N* HCl, 24 hours, 110° C.), this apparently homogeneous component disappeared and gave rise to 5 ninhydrin-positive components whose R_F values (in several solvents) were identical to those of authentic samples of glutamic acid, glycine, tyrosine, valine, and isoleucine (or leucine). Only the spot corresponding to tyrosine was radioactive. Since the *N*-terminal sequence of the *A*-chain of insulin is glycyl-isoleucyl-valyl-glutamyl-,[15] and since papain is known to hydrolyze readily peptide bonds involving a glutamyl residue,[16, 17] it may be inferred that papain had released from the labeled protein a fragment of the *A*-chain to which one or more glycyltyrosyl residues had become attached by peptide

linkage. It would appear, therefore, that the α-amino group of the *N*-terminal glycyl residue of insulin had participated in a cathepsin C–catalyzed transamidation reaction in which a C^{14}-labeled glycyltyrosyl residue had become "incorporated" into the protein.

Since the papain digest appeared to contain at least two radioactive components other than that discussed above, the possibility exists that, in addition to the *N*-terminal glycyl residue, other constituents of insulin (e.g., the *N*-terminal phenylalanyl residue of the *B*-chain) may have participated in replacement reactions. However, studies with synthetic replacement agents have indicated that, at pH values near 7.5, phenylalanyl peptides tend to be less efficient as replacement agents, and the ϵ-amino group of lysine (also present in the *B*-chain) is ineffective. Further studies on the composition of the two radioactive components of lower R_F may show, therefore, that they represent other fragments of the *N*-terminal sequence of the *A*-chain.

Labeling of Mitochondrial Proteins by Isotopic Tyrosine Derivatives.—The findings summarized above indicate that a proteolytic enzyme normally present in animal tissues can catalyze a transamidation reaction leading to the substitution of an α-amino group of a protein by an activated acyl group. Such reactions may occur in incubation mixtures used in the study of the incorporation of labeled "activated" amino acids into subcellular fragments. For this reason, experiments were undertaken on the incorporation of labeled amino acid residues into the proteins of rat liver mitochondria. The studies of Simpson and his associates[18] have shown that, in the presence of an ATP-generating system, the mitochondrial fraction of rat liver homogenates readily incorporates labeled amino acids into the mitochondrial protein. In the present experiments, an ATP-generating system was omitted, and the labeled compounds were the amides or acyladenylates of L-tyrosine and glycyl-L-tyrosine. Male rats (Sprague-Dawley) weighing about 60 gm. were killed, the livers were ground with 0.25 *M* sucrose in a loose glass homogenizer, and the mitochondrial fraction was separated by differential centrifugation in the manner described by Schneider and Hogeboom.[19] The mitochondria were washed four times with 0.25 *M* sucrose, the mitochondrial pellet being resuspended by homogenization between washings. The incubation mixture (1 ml.) contained: 5 μmoles of labeled compound; the mitochondrial suspension (0.1 ml., from about 0.2 gm. liver), which contained about 3 mg. protein; 50 μmoles tris(hydroxymethyl)aminomethane (pH 7.5); 5 μmoles KCl; 5 μmoles $MgCl_2$. All incubations were done in duplicate. After being shaken for the requisite time at 38° C., trichloroacetic acid was added, and the protein material was washed four times with cold 5 per cent trichloroacetic acid, once with hot (90° C.) trichloroacetic acid, and dissolved in 4 ml. 0.4 *N* NaOH. Unlabeled tyrosine or glycyltyrosine (25 μmoles in 0.5 ml.) was added to wash out adsorbed radioactivity, and the protein was reprecipitated with trichloroacetic acid and washed successively with ethanol, ethanol-ether, and ether.

C^{14}-labeled L-tyrosinamide was prepared from uniformly labeled L-tyrosine (31,000 cpm/μmole) by treatment of the ethyl ester with NH_3 in methanol[20]; m.p., 153°–154° C. When this compound was incubated with mitochondria under the conditions given in the preceding paragraph (total radioactivity in the incubation mixture, 155,000 cpm), the mitochondrial protein obtained became labeled, the extent of isotope "incorporation" increasing with longer periods of incubation.

The specific radioactivity obtained after an incubation period of 30 minutes was 15 cpm/mg; after 60 minutes, 42 cpm/mg; after 90 minutes, 96 cpm/mg. These findings may be compared with the result obtained by Simpson *et al.*[18] on the extent of labeling observed upon incubating mitochondria (from 1 gm. liver) with 0.3 μmole of C^{14}-leucine (500,000 cpm) in the presence of an ATP-generating system and the "pH 5 fraction" described by Hoagland *et al.*[8] After an incubation period of 20 minutes, precipitation of the mitochondrial protein in a manner similar to that described above gave a material having 20 cpm/mg protein. We are indebted to Dr. Simpson for permission to cite this result prior to the publication of the detailed account of his studies.

The fact that the labeling of mitochondrial protein by C^{14}-tyrosinamide involves an enzyme-catalyzed reaction is indicated by the finding that, if the mitochondrial fraction is heated for 3 minutes at 100° C., its ability to become labeled is lost. The protein obtained after 60 minutes incubation with such a heated preparation had 4 cpm/mg; after 90 minutes' incubation, 2 cpm/mg. It has long been known that animal tissues contain enzyme activity toward L-tyrosinamide;[21] in connection with the present studies, the ability of rat liver mitochondria to deamidate tyrosinamide was examined. Under the conditions of the incubation with the isotopic compound, L-tyrosinamide is deamidated to about 50 per cent in about 30 minutes, the reaction being complete at about 100 minutes. This enzymic activity may be attributed tentatively to an aminopeptidase of the type previously found in swine kidney.[21, 22]

Control experiments with C^{14}-labeled L-tyrosine showed that the observed labeling of the mitochondrial protein could not be attributed to adsorption of the amino acid formed by hydrolysis of the amide; it would appear that the method employed for the preparation of the mitochondrial protein for counting was adequate to remove such absorbed tyrosine. Samples of the incubation mixture treated with trichloroacetic acid immediately after mixing ("zero time incubation") gave samples of protein with negligible radioactivity (2 cpm/mg), indicating that essentially all the unchanged tyrosinamide that may have been adsorbed was removed by the precipitation method employed in these experiments.

A sample of the labeled mitochondrial protein obtained from experiments with C^{14}-labeled tyrosinamide was treated with 1-fluoro-2,4-dinitrobenzene at pH 9, and then hydrolyzed with 6 *N* HCl for 12 hours at 110° C. The hydrolyzate was extracted with ether, which readily dissolves dinitrophenylamino acids, including *O*,*N*-diDNP-tyrosine. It was found that about 90 per cent of the total radioactivity of the acid hydrolyzate was present in the ether extract, suggesting that nearly all of the C^{14}-tyrosine residues bound to the mitochondrial protein were located at *N*-terminal positions. One may tentatively conclude, therefore, that the "incorporation" of the isotopic tyrosyl residue had been effected by an enzyme-catalyzed transamidation reaction analogous to that discussed above in connection with the labeling of insulin by isotopic GTA. This conclusion is supported by the finding that the addition of 20 μmoles of hydroxylamine to the incubation mixture containing C^{14}-tyrosinamide reduces the extent of labeling of the mitochondrial protein in 60 minutes from 42 to 13 cpm/mg. In separate experiments, the enzymic formation of a hydroxamate could be demonstrated colorimetrically. The finding that most of the "incorporated" radioactivity appears to be associated with *N*-terminal

amino acids makes it unlikely that the tyrosyl residue had become attached to the side-chain carbonyl group of aspartic or glutamic acid in transamidation reactions involving asparaginyl or glutaminyl residues of the mitochondrial proteins.[23]

Initial experiments with C^{14}-labeled GTA indicate that its tyrosyl residue is "incorporated" into mitochondrial protein less effectively than is the case with C^{14}-tyrosinamide. The presence of cathepsin C in rat liver mitochondria has been reported,[24] and further studies with labeled GTA are needed to explain the difference in the extent of labeling observed with the two C^{14}-tyrosine derivatives.

Although the labeling of mitochondrial protein by C^{14}-tyrosinamide suggests that mitochondrial proteolytic enzymes can catalyze the transfer of aminoacyl groups to terminal amino acids of the protein, it cannot be concluded at present that α-amides of amino acids play a role in protein metabolism. However, as noted previously,[25] such amides are not so unphysiological as was once thought, and their metabolism merits closer investigation. A more general conclusion to be drawn from the experiment with C^{14}-tyrosinamide is that the demonstration of the labeling of a subcellular protein fraction by an isotopic amino acid cannot be considered to be evidence of protein synthesis, even if it is shown that the labeled amino acid is linked to the protein by a peptide bond. A more stringent criterion is the demonstration that the labeled amino acid appears at an interior position of the peptide chain of a reasonably well-defined single protein; an example of efforts in this direction is the work of Bates *et al.*[26] on the incorporation of labeled valine into the cytochrome c of liver mitochondria.

The work of numerous investigators[8-11] has led to the view that the "activation" of amino acids for protein synthesis involves a reaction with ATP, to form α-aminoacyladenylates. To compare the effectiveness of such compounds in the labeling of mitochondrial protein with that observed in the case of C^{14}-tyrosinamide, the corresponding C^{14}-tyrosyladenylate was synthesized. The synthesis involved the reaction of equimolar proportions of carbobenzoxy-L-tyrosine and adenosine-5′-phosphate in the presence of a slight excess of dicyclohexylcarbodiimide in a solution of tetrahydrofuran-water (1:1) containing 10 per cent pyridine. The carbobenzoxytyrosyladenylate could not be obtained in a state of analytical purity, because of the instability of the compound and since difficulty was encountered in separating it from adenosine-5′-phosphate. A less impure product was obtained by the same method in the reaction of carbobenzoxyglycyl-L-tyrosine with adenosine-5′-phosphate; calculated for $C_{29}H_{32}N_7O_{12}P$ (701.6), N 14.0, P 4.6; found, N 14.0, P 4.5. The tyrosine content (Folin-Ciocalteu method) of the latter product was found to be 26.3 per cent; theory, 25.7 per cent; the molar absorbance at 260 mμ in aqueous solution was 13,700. Both carbobenzoxytyrosyladenylate and carbobenzoxyglycyltyrosyladenylate reacted immediately with aqueous hydroxylamine or aqueous ammonia to form the expected hydroxamate or amide. These adenylates are rapidly hydrolyzed at acidic and alkaline pH values and are most stable in aqueous methanol near pH 7.

Immediately before use for experiments with rat liver mitochondria, the carbobenzoxy compounds were subjected to hydrogenolysis in methanol containing 5 equivalents of acetic acid, with freshly prepared palladium black as the catalyst. The concentration of tyrosyladenylate or of glycyltyrosyladenylate was determined by the hydroxylamine reaction, and 5 μmoles of the acyladenylate were added per

ml. of the incubation mixture. Control experiments showed that the adenosine-5′-phosphate present as an impurity did not affect the outcome of labeling experiments.

When 5 μmoles of a preparation of C^{14}-labeled tyrosyladenylate (12,000 cpm/μmole) were incubated with mitochondria under the conditions described above, the mitochondrial protein became labeled. After 10 minutes' incubation, the protein had 122 cpm/mg; after 30 or 60 minutes, 195 cpm/mg. The "zero time" incubation gave a protein sample having 2 cpm/mg. In the presence of 20 μmoles of hydroxylamine, the isotope incorporation into protein is completely abolished.

In contrast to the result with C^{14}-tyrosinamide, the labeling of mitochondrial protein by C^{14}-tyrosyladenylate cannot be considered to be an enzymic process, since mitochondria that had been heated for 3 minutes at 100° C. were labeled even more effectively. Under strictly comparable conditions, incubation of labeled tyrosyladenylate with heat-treated mitochondria for 30 minutes gave protein samples whose average radioactivity was 1,260 cpm/mg, instead of 195 cpm/mg found with unheated mitochondria. This six-fold increase in labeling is consistent with the view that tyrosyladenylate acted as a chemical acylating agent (like acetic anhydride) and that the denaturation of the mitochondrial proteins caused by the heat treatment made more groups (probably amino groups) available for non-enzymic acylation. Further evidence for such substitution of groups in the mitochondrial protein is provided by the result of treatment, with 1-fluoro-2,4-dinitrobenzene, of protein labeled in the incubation of unheated mitochondria with C^{14}-tyrosyladenylate. As in the experiment with labeled tyrosinamide, the acid hydrolyzate of the DNP-protein contained about 90 per cent of the radioactivity in the form of ether-extractable DNP-compounds, presumably *O*,*N*-diDNP-tyrosine.

If equimolar amounts (5 μmoles) of C^{14}-labeled tyrosinamide and of unlabeled tyrosyladenylate are incubated with liver mitochondria, the extent of labeling of the mitochondrial protein during a 60-minute incubation period is decreased from 42 to 24 cpm/mg, suggesting that the enzyme-activated tyrosyl residue from tyrosinamide and the reactive tyrosyl residue of tyrosyladenylate are competing for similar groups in the mitochondrial protein. It will be of interest to examine, with subcellular fractions, the competition between the tyrosyl residue of tyrosinamide and "activated" tyrosine formed from the free amino acid in the presence of an ATP-generating system and the "pH 5 fraction."

In view of the above findings, it is not surprising that C^{14}-labeled glycyltyrosyladenylate also causes labeling of the mitochondrial proteins, as do the carbobenzoxy derivatives of the two acyladenylates. For example, after incubation of mitochondria for 60 minutes with 5 μmoles of labeled carbobenzoxy-L-tyrosyladenylate (total radioactivity, 60,000 cpm/ml), the mitochondrial protein contained 196 cpm/mg.

While these experiments were in progress, we learned that Castelfranco, Moldave, and Meister had independently synthesized C^{14}-labeled glycyladenylate and tryptophyladenylate via the carbobenzoxy derivatives and had found that, when such compounds are incubated with the microsomal fraction of rat liver homogenates, the microsomal proteins become labeled. Moreover, when the microsomal fraction is heated before incubation with the labeled compounds, the extent of isotope "incorporation" is markedly increased, suggesting that non-enzymic acylation by

the highly reactive aminoacyladenylates is responsible for the labeling. We are grateful to Dr. Meister for permission to mention his results prior to their publication.

It should be emphasized that the experiments on the non-enzymic labeling of cellular proteins with synthetic aminoacyladenylates cannot be considered to disprove the hypothesis that such compounds are intermediates in the biosynthesis of proteins from amino acids. Obviously, the addition of a relatively large amount of an active acylating agent to a subcellular preparation is not equivalent to the coupled enzymic generation of such a compound and its rapid utilization in subsequent reactions catalyzed by a multi-enzyme system. The results reported in the present communication, however, indicate the need to take into account, in studies on the incorporation of labeled amino acids into cellular fragments, the possibility of non-enzymic acylation by reactive acid anhydrides that may be formed in biological systems.

Summary.—The intracellular proteinase cathepsin C catalyzes a transamidation reaction between C^{14}-labeled glycyl-L-tyrosinamide and insulin, leading to the labeling of the protein by substitution of an *N*-terminal amino acid residue. Similar transamidation reactions appear to be operative in the enzymic labeling of the proteins of rat liver mitochondria upon incubation of mitochondria with C^{14}-labeled tyrosinamide. The mitochondrial proteins may also be acylated by C^{14}-labeled L-tyrosyladenylate or glycyl-L-tyrosyladenylate, as well as by their *N*-carbobenzoxy derivatives; this process appears to be largely non-enzymic in nature, since it is promoted, rather than inhibited, by heat denaturation of the mitochondrial proteins.

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*THE MECHANISM OF CHEMICAL MUTAGENESIS. I. KINETIC STUDIES ON THE ACTION OF TRIETHYLENE MELAMINE (TEM) AND AZASERINE**

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Quantitative studies on the kinetics of chemical mutagenesis in bacteria have not paralleled in number investigations on radiation-induced mutations.[1] The work of Demerec and Hanson[2] on chemically induced mutagenesis in *Escherichia coli* illustrates a fruitful approach to this problem and had its origin in two developments: the discovery of a mutagen of relatively low toxicity—manganous chloride—and the introduction of a simple and efficient assay system for measuring the mutation rate from streptomycin dependence to independence. The main emphasis in the work of the Demerec group,[3–4] however, subsequently shifted from quantitative studies of a particular mutational system to the evaluation of the specificity of several mutagens on a variety of genetic loci.

The present work represents an examination of two other potent mutagens—triethylene melamine (TEM) and azaserine— which are active over wide ranges of concentration and temperature without excessive killing. This study was prompted by an awareness of the advantages of a mutagenic system which would lend itself to quantitative kinetic studies of chemically induced mutational processes and thus provide a tool for deeper insight into the mechanism of events leading to a mutation.

MATERIALS AND METHODS

The streptomycin-dependent (*sd-4*), cysteine-requiring (*cys-2*) strain Sd4-73 of *E. coli* was obtained from Dr. M. Demerec. The stock culture was periodically purified by selection from among a number of single-colony isolates on the basis of a low proportion of spontaneous back-mutants.[5] Nutrient broth (Difco) containing 20 μg/ml of streptomycin was employed for preparing liquid cultures. Three solid media served for enumeration of the total number of colonies and the proportion of respective mutants: Difco nutrient agar with and without 100 μg/ml of streptomycin, and minimal agar (7 gm. K_2HPO_4; 2 gm. KH_2PO_4; 0.5 gm. Na_3 citrate$\cdot 5H_2O$; 0.1 gm. $MgSO_4\cdot 7H_2O$; 1 gm. $(NH_4)_2SO_4$; 2.5 gm. glucose; 20 gm. agar) with 100 μg/ml streptomycin.

In the general procedure, an overnight culture of Sd4-73 was prepared by incubation with forced aeration at 32° C. The cells were centrifuged, washed twice, and finally suspended in distilled water, to which the desired amount of mutagen had been added prior to incubation at a particular temperature for a chosen period of time. After mutagen treatment, the cells were washed free of the mutagen,